

# Bradford reactive soil protein in Appalachian soils: distribution and response to incubation, extraction reagent and tannins

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**Abstract** Bradford reactive soil protein (BRSP) is thought to correlate to glomalin, an important soil glycoprotein that promotes soil aggregate formation and may represent a significant pool of stable soil organic matter (SOM). However, more information is needed about its importance in Appalachian soils and its relationships with other soil properties. We measured BRSP in 0–20 cm soil from pastures, hayfields, cultivated fields or forest areas in southern West Virginia. Highest amounts of BRSP were found near the soil surface and decreased significantly with depth for all land uses except cultivated sites. Forest and pasture sites contained more BRSP than hayfields or cultivated fields but these differences occurred only in the 0–5 cm depth. Overall averages of C and N in BRSP represented about 4.0 and 6.5% of the total soil C and N respectively. During a 395 day soil incubation, we found CO<sub>2</sub>–C evolution rates comparable to other studies but only small changes in BRSP (<10%) including some evidence for increases during incubation. Sodium citrate, sodium pyrophosphate, and sodium oxalate recovered significantly more BRSP from soil than the other extractants we tested with highest

extraction efficiencies observed for sodium citrate and pyrophosphate. Recovery of BRSP appears related to negative charge and buffering capacity of both the soil and extractant. Extractants with low negative charge had little buffering capacity and yielded little BRSP. Tannic acid appeared to increase extraction of BRSP but less soluble-N was recovered from tannin-treated samples than from untreated controls and E4/E6, the ratio of absorbance at 465 and 665 nm, decreased, evidence for the formation of larger or heavier molecules. Formation of dark-colored substances during extraction suggests the colorimetric Bradford assay may overestimate soil protein when tannins are present. Recovery of less soluble-N from soil extracts and lower E4/E6 ratios suggests tannins may bind with soil constituents themselves or form non-extractable N-containing complexes.

**Keywords** Glomalin · Bradford reactive soil protein · BSRP · Extractants · Tannins · Soil carbon · Soil nitrogen

## Introduction

The importance of arbuscular mycorrhizal fungi (AMF) to ecosystem function is increasingly appreciated (Bever et al. 2001; Rillig 2004a,b; Staddon 2005; Zhu and Miller 2003) including

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their role in agriculture (Jeffries et al. 2003; Ryan and Graham 2002; Sen 2003). Glomalin, a recently described glycoprotein product of AMF is of particular interest because it is associated with the formation of soil aggregates and thought to be a significant pool of decomposition resistant organic matter (Haddad and Sarkar 2003a; Nichols 2003; Rillig et al. 2001b; Wright and Upadhyaya 1996; 1998). Glomalin is not easily extracted from soil, and protein, determined by the Bradford assay of autoclaved sodium citrate-soil extracts, has been operationally or methodologically defined to correspond to easily extractable or total glomalin (Rillig 2004a; Wander 2004; Wright and Jawson 2001; Wright and Upadhyaya 1996). Bradford reactive soil protein (BRSP) by itself or together with information from enzyme-linked immunosorbent assays has been used to quantify stocks and turnover rates of glomalin and infer functional relationships between glomalin and soil properties. Typical concentrations of BRSP range from 2 to 15 mg g<sup>-1</sup> soil (Nichols 2003) but much higher values have been reported for older soils in Hawaiian chronosequences (Rillig et al. 2001b) and lower values are generally observed in more arid sites (Bird et al. 2002; Rillig et al. 2001a; Wright and Upadhyaya 1998).

Information about pools of glomalin in Appalachian agroecosystems and forested areas is needed because establishment of mixed land-uses such as silvopastures may include removal or thinning of existing trees, establishment and maintenance of forage swards, and the effects of grazing animals. These activities physically disturb soil, modify microclimate and plant-soil interactions, and change soil chemistry through additions of lime, plant nutrients and animal wastes. Comparatively high concentrations of glomalin are associated with conditions that might be expected to favor AMF activity (Ryan and Graham 2002) or diversity (Helgason et al. 1998) such as management regimes that minimize physical disruption of the soil by tillage (Borie et al. 2000; Wright and Anderson 2000; Wright et al. 1999) or low to moderate soil fertility (Lovelock et al. 2004a; Treseder 2004; Treseder and Allen 2002). Patterns of BRSP are less clearly affected by other types of disturbance such as fire or animal grazing. Wuest et al. (2005) found

annual burning reduced BRSP in dryland wheat fields in eastern Oregon but Knorr et al. (2003) found no effect of annual burning on immunoreactive portions of BRSP in forested watersheds in southern Ohio. Grazing by cattle does not appear to reduce BRSP in soil (Franzluebbers et al. 2000).

More information is also needed about the role glomalin plays in nutrient cycling and its relationship to other pools of organic matter in part because some of the glomalin represented by BRSP is thought to resist decomposition (Lutgen et al. 2003; Nichols 2003; Rillig et al. 2003; Rillig et al. 2001b). Concentrations of BRSP accumulate in soil with site or stand age but decrease with depth (Franzluebbers et al. 2000; Harner et al. 2004; Rillig et al. 2001b; Staddon 2005), tend to be highly correlated with soil C and N (Bird et al. 2002; Haddad and Sarkar 2003b; Rillig et al. 2003; Wuest et al. 2005) and can sequester toxic metals (Gonzalez-Chavez et al. 2004).

Glomalin like other soil organic matter (SOM) may be impacted by inputs of plant secondary compounds. Phenolic plant secondary compounds such as tannins are abundant, accounting for up to 40% of the composition of leaves and bark of some species, and are known to influence a number of soil processes that make them particularly important elements in the management of silvopastoral ecosystems (Appel 1993; Fierer et al. 2001; Hattenschwiler and Vitousek 2000; Kraus et al. 2003a). Phenolics influence soil biogeochemistry, depending in part upon specific structural characteristics (Fierer et al. 2001; Kraus et al. 2003b), because they can form complexes with many classes of biologically important molecules including carbohydrates, proteins, polysaccharides, bacterial cell membranes, and enzymes which affect decomposition rates and nutrient cycling (Hagerman 2002; Hattenschwiler and Vitousek 2000; Kraus et al. 2003a). Formation of complexes by tannins in soil or litter might affect recovery or quantification of BRSP.

The overall goal of our research was to determine if BRSP represents a significant pool of recalcitrant organic matter in Appalachian agroecosystems. The specific objectives of this study were to: (a) measure the quantity and distribution of BRSP with depth and land use and estimate its

contribution to total soil-C and -N pools; (b) determine changes in BRSP after lab incubation; (c) compare the efficacy of different extracts on the recovery of BRSP; and (d) determine if important plant secondary compounds like tannins can bias recovery of BRSP in Appalachian soils. We hypothesized that adding tannins to soil would decrease recovery of BRSP.

## Materials and methods

### Study location and plots

We sampled 44 locations distributed in among 13 farm units in Southern West Virginia. Locations were grouped into primary land-use classes; cultivated (cultivated crops including long term family gardens), pastures, hayfields, and forest (mixed deciduous or pine). Soils were generally fine or sandy loams on slight to moderate slopes (Table 1).

We also selected a subset of samples from 5 farms, each with samples of forest, pasture, and cultivated land to compare the efficiency of different soil extractants, see if tannins would affect recovery of BRSP and to estimate the C and N content of BRSP, (1, 5, 6, 11, and 13 in Table 1).

### Soil sampling

At each location, 10 soil cores (6.35 cm in diameter) were collected along one or two transects at 50 foot intervals. Cores were subdivided in the field into 0–5, 5–10, and 10–20-cm depth increments and bulked. In the lab composite samples were sieved (2 mm), dried at 55°C and stored until further analysis. These soil samples were analyzed for pH and electrical conductivity (EC) using a 1:1 DI soil-water paste (Rhoades 1996; Thomas 1996).

### Bulk density

At each site, soil bulk density was determined separately for each depth increment from duplicate samples collected using a hand-held coring device. Moisture content of the cores was determined by the gravimetric method, and bulk

density or the dry mass per unit volume was calculated (Blake and Hartge 1986).

### Soluble-C and -N (SOC, SN)

Soluble organic carbon (SOC) and nitrogen (SN) were extracted from sieved, dry samples using a sequential extraction procedure similar to Ghani et al. (2003). Samples were first extracted with cool DI water (23°C). After shaking for 30 min, samples were centrifuged (3 min at 17,000 g), decanted and analyzed with a Shimadzu TOC-VCPN analyzer equipped with a TNM-1 module (Shimadzu Scientific Instruments, Columbia, MD). More water was added to soil samples, vortexed, incubated (80°C for 16 h) in a hot water bath and re-assayed. Soluble-C and -N recovered with cool water is thought to be related to recent inputs into soil such as fertilizer, lime, manure, or soluble plant residues while C and N recovered after hot water incubation is positively correlated with soil microbial biomass-C and -N, mineralizable N and total carbohydrates (Ghani et al. 2003). For this work, cool and hot water extract data were pooled together before further analysis.

### Soil organic matter and total-C and -N

We also determined whole SOM using the loss on ignition (450°C for 4 h) method similar to that described by Cambardella et al. (2001). We determined total soil-C and -N content by dry combustion (Nelson and Sommers 1996) using a Carlo Erba analyzer (EA1108 CHNSO, Milan, Italy).

### Bradford reactive soil protein

We extracted total BRSP from soil samples following the methods of suggested by Wright and Upadhyaya (1996, 1998) and used in subsequent studies as an operational definition of total glomalin (Harner et al. 2004; Lovelock et al. 2004a, b; Nichols 2003; Rillig et al. 2003; Wander 2004). Briefly, 1 g. samples of soil were autoclaved (128°C) for 60 min in 8 ml of 50 mM citrate buffer (adjusted to pH = 8.0) centrifuged, and the supernatant containing glomalin was collected. The extraction process was repeated six times and

**Table 1** Locations and sample descriptions for Forest (F), Pature (P), Hayfied (H), and Cultivated (C) sites from 13 farm units in Southern West Virginia

Site	Location	Samples collected				Soils represented
		F	P	H	C	
1	N 37°54.1' W 80°21.0'	TIC	TIB	Cmb (alfalfa) Cmc (fescue)	TIC (corn) Cmb (corn)	TIB, TIC (Teas-Calvin-Litz silt loam, 3–8 and 8–15 % slope respectively): Fine-loamy, mixed, active, mesic Ultic Hapludalfs-Loamy-skeletal, mixed, active, mesic Typic Dystrudepts-Loamy-skeletal, mixed, active, mesic Ruptic-Ultic Dystrudepts. Cmb, Cmc (Clymer fine sandy loam, 3–10 and 10–20 % slope respectively): Coarse-loamy, siliceous, active, mesic Typic Hapludults, mixed, semiactive, mesic Typic Paleudults. FkC (Frederick cherty silt loam, karst 8–15% slope): Fine, mixed, semiactive, mesic Typic Paleudults DfB (Duffield silt loam, 3–10% slope): Fine-loamy, mixed, active, mesic Ultic Hapludalfs.
2	N 37°46.4' W 80°37.5'	FkC	FkC	FkC		
3	N 37°35.6' W 80°31.6'		DfB	DfB		
4	N 37°34.8' W 80°26.8'	FcC	FcC	FcC		FcC (Frederick cherty silt loam, 8–15% slope): Fine, mixed, semiactive, mesic Typic Paleudults.
5	N 37°33.5' W 80°22.3'	CbE	CbE MuC		MuC	CbE (Chilhowie-Tumbez very rocky silty clay, 25–45% slope): Very-fine, mixed, mesic Typic Hapludalfs-Fine, mixed, active, mesic Typic Hapludalfs. MuC (Murrill channery loam, 3–8% slope): Fine-loamy, mixed, semiactive, mesic Typic Hapludults.
6	N 37°39.5' W 80°57.4'	GaC	GaC	GaC	GaC	GaC (Gilpin silt loam, 8–15% slope): Fine-loamy, mixed, active, mesic Typic Hapludults.
7	N 37°58.0' W 80°48.5'	DbB		DbB	DbB	DbB (Dekalb fine sandy loam, 5–12% slope): Loamy-skeletal, siliceous, active, mesic Typic Dystrudepts.
8	N 37°58.8' W 80°47.9'	GIC	GIC			GIC (Gilpin silt loam, 10–20% slope): Fine-loamy, mixed, active, mesic Typic Hapludults.
9	N 37°45.6' W 81°25.1'	MkC	MkC	MkD		MkC (Muskingum silt loam, 10–20% slope): Loamy-skeletal, mixed, active, mesic Typic Dystrudepts.
10	N 37°47.6' W 80°58.4'	DsC	DsC	DsC		DsC (Dekalb and Gilpin very stony soils, 5–20% slope): Loamy-skeletal, siliceous, active, mesic Typic Dystrudepts-Fine-loamy, mixed, active, mesic Typic Hapludults
11	N 37°45.2' W 80°58.8'	GIC	GIC	GIC	GIC	GIC (Gilpin silt loam, 10–20% slope): Fine-loamy, mixed, active, mesic Typic Hapludults.
12	N 37°32.9' W 80°31.6'		FsC	FsC	FsC	FsC (Fredrick and Dunmore very rocky soils, 3–15% slope): Fine, mixed, semiactive, mesic Typic Paleudults-Clayey, kaolinitic, mesic Typic Paleudults.
13	N 37°49.7' W 80°43.2'	TIC	TIC	TIC	TIC	TIC (Teas-Calvin-Litz silt loam, 8–15% slope): Fine-loamy, mixed, active, mesic Ultic Hapludalfs-Loamy-skeletal, mixed, active, mesic Typic Dystrudepts-Loamy-skeletal, mixed, active, mesic Ruptic-Ultic Dystrudepts.

pooled extracts were centrifuged to remove soil particles, and protein concentration was determined by the Bradford assay using bovine serum albumin as standards (Bradford 1976). Values of BRSP are expressed as gravimetric concentrations (mg BRSP per g soil) or converted to a volumetric concentration by multiplying by the soil BD.

#### Soil incubation

We measured CO<sub>2</sub> evolution and changes to BRSP content of 10 g. soil samples during a 395-day static incubation following the methods outlined by Zibilske (1994). Each moisture adjusted soil sample (50% water filled pore space) was incubated in 1 l sealed canning jars in the dark at 23°C. Samples were kept moist by a small amount of water placed in the bottom of the jar. Carbon mineralization was measured as CO<sub>2</sub> evolved during the incubation and trapped in beaker containing a 1 M NaOH solution. Periodically, canning jars were opened and the NaOH traps were removed and capped. Jars were flushed with air and resealed. CO<sub>2</sub>-C trapped in NaOH was determined by colorimetric titration with 0.5 M HCl. At the end of the incubation period we extracted total BRSP from soil for comparison to preincubation values.

#### Effects of different extractants and tannins

We extracted BRSP from a subset of samples (see above) using sodium citrate and five other extractants: sodium pyrophosphate, sodium oxalate, sodium acetate, sodium formate, and sodium orthophosphate all at 50 mM concentration and adjusted to pH 8.0. The extraction process was repeated six times but extracts from each cycle were analyzed separately for BRSP. Data were used to determine patterns of cumulative BRSP recovery for each extractant and to determine proportion of total BRSP extracted by the first cycle of autoclaving as an index of extract efficiency.

We determined the effects of model tannins on recovery BRSP after shaking 1 g. soil samples in 4 ml H<sub>2</sub>O for 30 min, together with 10 mg of hydrolyzable (tannic acid, Fisher Chemical ACS

reagent grade, MW 1701.2) or condensed (quebracho, UNITAN) tannin prepared from the heartwood of Quebracho Colorado trees (*Schinopsis balansae* and *Schinopsis lorentzii*) which grow in South America. After shaking, 4 ml of 50 mM sodium citrate (pH 8.0) was added to samples before the first extraction. Five subsequent extractions used 8 ml of 50 mM concentration adjusted to pH 8.0. We determined total BRSP and the amount of soluble-N in extracts as outlined above and determined the ratio of absorbance at 465 and 665 nm in BRSP extracts (E4/E6 ratio) with a Shimadzu UV1700 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Observation of decreasing E4/E6 ratios is associated with the formation of larger or heavier molecules (Chen et al. 1977).

#### C and N in BRSP extracts

Bradford reactive soil protein from the 0–5 to 10–20 cm sodium citrate extracts was analyzed for total C and N content following Nichols (2003). Sodium citrate extracts ( $N = 22$ ) were precipitated with concentrated (12 M) HCl, redissolved in 1.0 M NaOH, dialyzed against DI water (Spectra/Por 6 Membrane MWCO: 10,000 flat width 45 mm), lyophilized, and analyzed for C and N with a FlashEA 1112 NC Analyzer (CE Elantech, Lakewood, NJ). Results are reported as a percent of gravimetric weight of the lyophilized material.

Where appropriate, models relating C or N content of lyophilized BSRP to colorimetrically determined estimates of BSRP in soil were determined in SYSTAT 11 using the Least Median of Squares (LMS) method of estimation variables (SYSTAT Software Inc. 2004). We selected this method because it minimizes the median of the squared residuals and is robust with respect to outliers in Y as well as outliers in X-space.

#### Data analysis

Data were analyzed by analysis of variance (ANOVA) using SAS 8.02 and PROC MIXED to determine differences related to treatment and depth using a model that featured fixed effects of

land use and depth as a repeated measure (Littell et al. 1996; SAS 1999). The KR (Kenward-Roger) option was used to calculate degrees of freedom and the covariance structure was evaluated using Akaike's Information Criterion, a means for choosing between competing statistical models. For most variables, we used the ARH(1) (heterogeneous first-order autoregressive) covariance structure. For all analyses, multiple pairwise comparisons of means were performed using Tukey-Kramer adjusted tests when the main treatment comparisons were found significant. A value of 5% was selected as the minimum criterion for significance unless otherwise noted. Significant interactions were determined using the SLICE option. The SLICE option allows tests of significance of one effect at each level of another effect. Where appropriate, data was  $\log_{10}$  or arcsine transformed to reduce heteroscedasticity before analysis. Mean values shown in the text are arithmetic values followed by the standard error in parenthesis. Because treatments were applied to matched samples we also compared changes to BRSP concentration after incubation and effects of tannins on BRSP recovery with adjusted (Bonferroni) paired-*t* tests and the non-parametric sign test and calculated Bonferroni corrected correlation coefficients between BRSP and selected soil variables with SYSTAT 11 (SYSTAT Software Inc. 2004).

## Results

Patterns of BRSP and selected soil properties across land-use types and soil depth

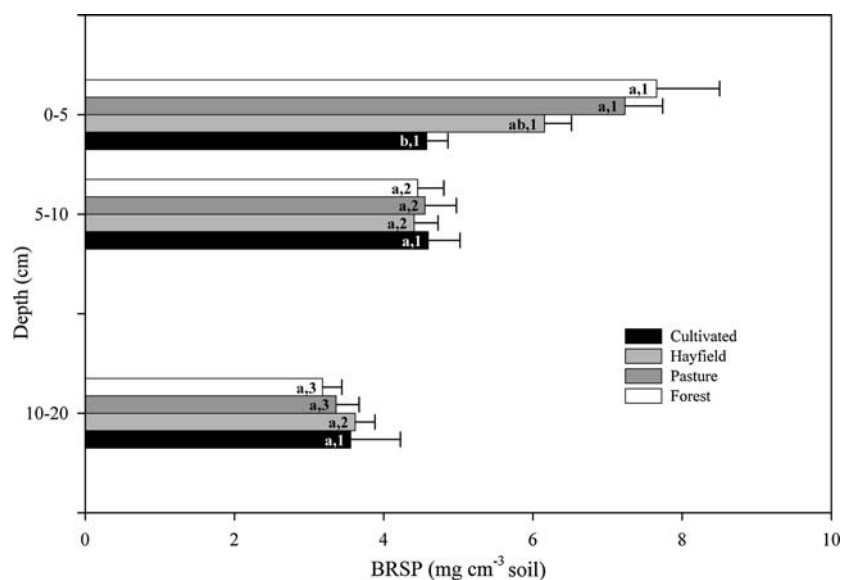
Analysis of variance revealed a significant interaction between land use and soil depth on volumetric concentrations of BRSP ( $P < 0.001$ ). Average BRSP ( $\text{mg cm}^{-3}$  soil) differed significantly among land uses only at the 0–5 cm depth where concentrations for forest sites were about 65% higher than the lowest in cultivated sites (Fig. 1). Concentrations of BRSP decreased with depth in all land uses except cultivated.

There was also a significant interaction between land-use and depth for bulk density (BD) ( $P < 0.01$ ). Lower average values of BD were observed for forest sites than other land uses at the soil surface (0–5 cm) but there were no difference among land uses at the other depths (Table 2). Average BD increased with depth for all land uses except cultivated sites.

Significant main effects of land-use ( $P < 0.0001$ ) and depth ( $P = 0.01$ ) were observed on pH. The average pH for forests was at least 1.4 pH units lower than other land uses and was highest, near 6.0, for cultivated sites. There was a small but significant decrease in pH with depth.

A significant interaction between land-use and depth was observed on electrical conductivity

**Fig. 1** Mean (SE) values of total Bradford reactive soil protein (BRSP) expressed on a volumetric basis for Forest ( $n = 11$ ), Pasture ( $n = 13$ ), Hayfield ( $n = 12$ ), and Cultivated ( $n = 7$ ) sites. Within each depth, differences among land uses are denoted by letters. For each land use, differences among depths are denoted by numbers (Tukey's HSD,  $P < 0.05$ )



**Table 2** Mean (SE) soil bulk density (BD), pH, electrical conductivity (EC), water-soluble and total soil-C and -N, and contribution of Bradford reactive soil protein (BRSP) to total soil pools

Depth (cm)	Land use	Soil BD <sup>a</sup> (g cm <sup>-3</sup> )	pH	EC <sup>a</sup> ds cm <sup>-3</sup>	Soluble soil <sup>a</sup>		Total soil <sup>a</sup>		BRSP % of soil pool <sup>b</sup>	
					C µg cm <sup>-3</sup>	N	C (mg cm <sup>-3</sup> )	N	C	N
0–5	Forest	0.93b,2 (0.05)	4.3	110.1b,1 (11.6)	2433.8a,1 (255.3)	233.7b,1 (33.4)	58.8a,1 (5.6)	4.8ab,1 (0.7)	5.4 (0.6)	6.9 (0.5)
	Pasture	1.13a,3 (0.02)	5.5	205.0a,1 (29.1)	2546.1a,1 (252.4)	402.3a,1 (45.2)	53.2a,1 (3.7)	5.3a,1 (0.4)	4.8 (0.3)	5.5 (0.2)
	Hayfield	1.20a,2 (0.03)	5.7	139.3ab,1 (12.0)	1837.1ab,1 (138.0)	265.2ab,1 (20.4)	40.3ab,1 (2.7)	3.8ab,1 (0.3)	4.9 (0.4)	6.8 (0.7)
	Cultivated	1.24a,1 (0.04)	5.9	166.9ab,1 (27.8)	1237.6b,1 (140.6)	197.5b,1 (28.1)	28.6b,1 (2.0)	2.9b,1 (0.2)	4.2 (0.2)	6.5 (0.4)
5–10	Forest	1.18a,1 (0.03)	3.9	57.2b,2 (5.2)	1219.5a,2 (100.0)	101.6b,2 (7.7)	32.0a,2 (1.9)	2.5a,2 (0.2)	3.7 (0.3)	7.3 (0.4)
	Pasture	1.25a,2 (0.02)	5.4	100.9a,2 (11.9)	1314.6a,2 (121.1)	196.8a,2 (22.0)	31.1a,2 (2.6)	3.3a,2 (0.3)	3.8 (0.2)	5.5 (0.2)
	Hayfield	1.28a,12 (0.03)	5.5	86.5ab,2 (8.1)	1057.3a,2 (64.2)	144.9ab,2 (9.9)	27.1a,2 (2.0)	2.7a,2 (0.2)	4.2 (0.3)	6.9 (0.6)
	Cultivated	1.29a,1 (0.03)	6.0	141.0a,1 (23.3)	1062.3a,1 (124.5)	167.7ab,12 (27.8)	27.1a,1 (2.9)	2.7a,1 (0.3)	4.4 (0.2)	7.0 (0.7)
10–20	Forest	1.28a,1 (0.04)	3.9	42.0b,3 (3.1)	704.6a,3 (46.05)	60.2b,3 (3.8)	24.0a,3 (3.8)	2.1a,2 (0.4)	3.2 (0.3)	7.0 (0.8)
	Pasture	1.35a,1 (0.03)	5.3	69.8b,3 (6.9)	767.3a,3 (62.5)	104.6a,3 (10.5)	20.8a,3 (1.6)	2.2 a,3 (0.1)	3.6 (0.2)	6.0 (0.3)
	Hayfield	1.39a,1 (0.03)	5.5	68.8b,3 (6.1)	768.9a,3 (34.4)	98.3a,3 (7.0)	22.3a,2 (1.5)	2.2a,2 (0.2)	3.7 (0.3)	7.0 (0.7)
	Cultivated	1.40a,1 (0.05)	5.9	134.5a,1 (22.0)	802.9a,2 (95.7)	129.9a,2 (21.8)	23.2a,1 (4.5)	2.3 a,1 (0.3)	3.4 (0.2)	6.1 (0.5)
All uses	0–5 cm	1.12 (0.02)	5.3A	156.4 (11.9)	2106.5 (129.5)	287.6 (21.0)	47.0 (2.5)	4.4 (0.3)	4.9A (0.2)	6.4 (0.3)
	5–10 cm	1.25 (0.01)	5.2B	93.3 (7.2)	1177.4 (53.2)	153.2 (10.1)	29.6 (1.2)	2.8 (0.1)	4.0B (0.1)	6.6 (0.3)
	10–20 cm	1.35 (0.02)	5.1B	74.3 (6.6)	757.3 (27.9)	95.4 (6.2)	22.4 (1.3)	2.2 (0.1)	3.5C (0.1)	6.6 (0.3)

**Table 2** continued

Depth (cm)	Land use	Soil BD <sup>a</sup> (g cm <sup>-3</sup> )	pH	EC <sup>a</sup> ds cm <sup>-3</sup>	Soluble soil <sup>a</sup>		Total soil <sup>a</sup>		BRSP % of soil pool <sup>b</sup>	
					C µg cm <sup>-3</sup>	N	C (mg cm <sup>-3</sup> )	N	C	N
All depths	Forest	1.13 (0.03)	4.0B (0.1)	69.8 (6.7)	1452.6 (156.4)	131.8 (17.2)	38.3 (3.5)	3.1 (0.3)	4.1 (0.3)	7.0 (0.3)
	Pasture	1.24 (0.02)	5.4A (0.1)	125.2 (14.0)	1563.1 (154.8)	238.0 (26.6)	35.4 (2.7)	3.7 (0.3)	4.1 (0.2)	5.7 (0.1)
	Hayfield	1.29 (0.02)	5.6A (0.1)	98.2 (7.2)	1221.1 (91.5)	169.4 (14.2)	29.9 (1.8)	2.9 (0.2)	4.3 (0.2)	6.9 (0.4)
	Cultivated	1.31 (0.03)	5.9A (0.1)	147.5 (13.8)	1034.3 (77.7)	165.1 (15.6)	26.3 (1.9)	2.6 (0.2)	4.0 (0.2)	6.5 (0.3)

BRSP-C and -N were calculated using values from Fig. 3. Within each depth, differences among land uses are denoted by letters. For each land use, differences among depths are denoted by numbers (Tukey–Kramer HSD;  $P < 0.05$ ). Sample numbers for forest, pasture, hayfield and cultivated sites were 11, 13, 12, and 7–8, respectively

<sup>a</sup> ANOVA calculated with  $\log_{10}$ -transformed data

<sup>b</sup> ANOVA calculated with arcsine-transformed data

(EC) ( $P < 0.0001$ ). The highest average value of EC occurred in pasture sites at the soil surface (0–5 cm), in cultivated sites at other depths, and was lowest in forest sites at all depths. Average EC decreased with depth for all land uses except cultivated.

Significant interactions between land use and soil depth were observed for soluble-C and -N ( $P < 0.0001$ ). Average concentrations of soluble-C and -N, differed among land uses in the 0–5 cm depth where pasture sites contained about twice the amount of cultivated sites. In forest sites, soluble-C was also relatively high but soluble-N was comparatively low. At other depths, soluble-C did not differ among land uses but volumetric concentrations of soluble-N were lower for forest sites than other land uses. Concentrations of both soluble-C and -N decreased with depth for all land uses.

Significant interactions between land use and soil depth were also observed for total soil-C ( $P < 0.001$ ) and -N ( $P < 0.001$ ). In both, differences among land uses occurred only in the 0–5 cm depth with greatest volumetric concentration in pasture sites and lowest in cultivated sites. Concentrations of total-C and -N decreased with depth for forest, pasture and hayfield sites but not for cultivated locations (Table 2). The C/N ratio varied among land uses but not with depth, the average for forest sites, 12.9 (0.5), was greater than hayfields, 10.4 (0.3), cultivated 10.2 (0.3) or pasture sites 9.7 (0.2).

Bradford reactive soil protein was negatively correlated with soil bulk density but positively correlated with EC (Table 3). Especially strong positive correlations were observed between BRSP and SOM, Total Soil-C and -N and Soluble Organic-C and -N. Conversely, BRSP was not correlated to soil pH or C:N ratios.

#### Relation of BRSP concentrations to soil carbon and nitrogen pools

The concentration of carbon, but not nitrogen, increased in lyophilized BRSP material as a function of gravimetric BRSP concentrations determined colorimetrically (Fig. 2). The LMS model for carbon was % C =  $13.9 + 3.26 \text{ BRSP}$ ,  $r^2 = 0.79$ . The average N concentration in lyophilized BRSP

**Table 3** Pearson correlation coefficients and Bonferroni adjusted probabilities of Bradford reactive soil protein (BRSP) and other soil properties across all land uses and depths ( $N = 132$ )

Soil variable	BRSP	
	Pearson correlation coefficient	Bonferroni corrected probability
Bulk density	-0.654	< 0.0001
pH	0.001	NS
EC	0.458	< 0.0001
SOM	0.930	< 0.0001
Total soil-C	0.943	< 0.0001
Total soil-N	0.896	< 0.0001
Total C:N	0.196	NS
Soluble organic-C	0.885	< 0.0001
Soluble organic-N	0.775	< 0.0001
Soluble C:N	0.011	NS

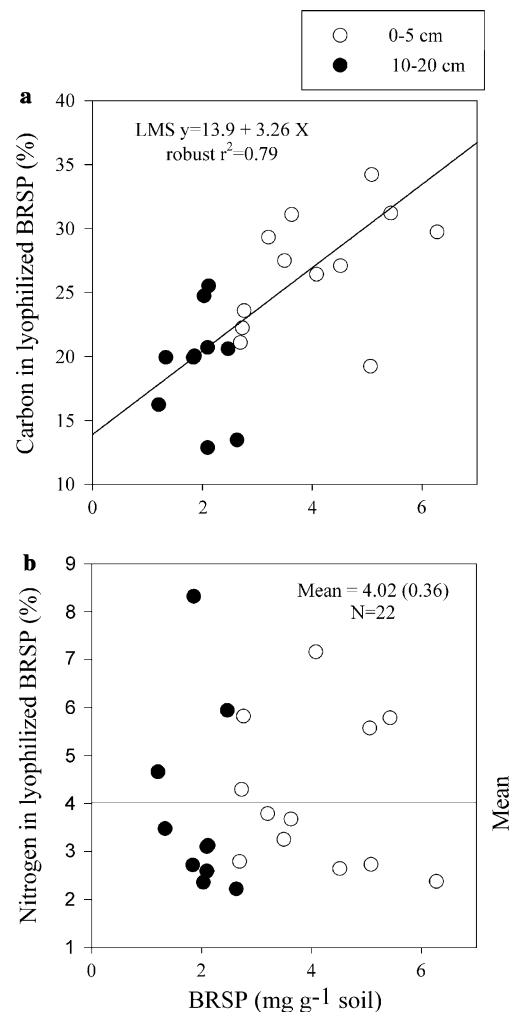
was 4.02% (0.36). These values were used to estimate BRSP-C and -N in relation to total soil-C and -N (Table 2).

The contribution of BRSP-C to total soil-C pools decreased significantly with depth ( $P < 0.0001$ ), from near 5% at 0–5 cm to 3.5% at 10–20 cm, but did not vary among land uses. Contributions of BRSP-N to total soil stocks did not vary with either land use or depth, averaging 6.51% (0.16) with a range of values from 2.9 to 13.8%.

#### Changes in BRSP after soil incubation

After nearly 400 days of incubation, patterns for cumulative  $\text{CO}_2\text{-C}$  evolution and gravimetric BRSP concentrations mirrored each other and resembled those observed for volumetric concentrations of BRSP in fresh soil (Table 4, Fig. 1). A significant interaction was found between land use and soil depth for average  $\text{CO}_2\text{-C}$  evolution ( $P < 0.0001$ ) and BRSP ( $P < 0.0001$ ) with differences among land uses only at the 0–5 cm depth where the highest respiration rates and BRSP concentrations, observed for forest sites, were more than twice that of the lowest values in cultivated sites. Cumulative  $\text{CO}_2\text{-C}$  evolution decreased significantly with depth for all land uses while BRSP decreased for all land uses except cultivated (Table 4).

A significant interaction was also found between land use and soil depth for % C remain-

**Fig. 2** (a) Carbon and (b) Nitrogen composition of lyophilized BRSP in relation to concentrations of BRSP in soil determined colorimetrically

ing after incubation ( $P < 0.05$ ) due mainly to variations among depths observed for hayfields, there were no differences among land-uses at any depth and no differences among depths for other land uses. Though 0–5 cm samples evolved more than twice the amount of  $\text{CO}_2\text{-C}$  as 10–20 cm samples during the incubation period, both accounted for a comparable proportion of the total soil C pool.

A significant main effect of depth was observed for changes to BRSP concentrations ( $P < 0.0001$ ) and % BRSP remaining ( $P < 0.0001$ ) after the 395 day incubation period. The largest absolute changes to BRSP concentration were observed for

0–5 cm samples but these amounted to losses of less than 10% of the beginning BRSP pool. Conversely, at 10–20 cm, significant increases in BRSP concentrations were observed equivalent to about

7% of the initial BRSP pool. Changes in BRSP during incubation, though small, were significantly greater than zero as determined by paired-*t* and non-parametric sign tests (Table 4).

**Table 4** Mean (SE) cumulative CO<sub>2</sub>-C respired, % soil-C remaining, Bradford reactive soil protein (BRSP), and percent of initial BRSP pools after a 395 day incubation

Depth (cm)	Land use	CO <sub>2</sub> -C respired during incubation <sup>a</sup> (mg g <sup>-1</sup> soil)	Soil-C after incubation <sup>a</sup> (%)	BRSP after incubation <sup>a</sup> (mg g <sup>-1</sup> soil)	change in BRSP <sup>a,b</sup> (mg g <sup>-1</sup> soil)	BRSP after incubation <sup>a</sup> (% of initial pool)
0–5	Forest	5.06a,1 (0.26)	91.6a,1 (0.7)	7.55a,1 (0.77)	-0.69 (0.32)	92.3 (4.2)
	Pasture	3.84ab,1 (0.28)	91.8a,1 (0.3)	5.74ab,1 (0.34)	-0.69 (0.21)	90.3 (2.7)
	Hayfield	3.45bc,1 (0.35)	89.9a,2 (0.7)	4.86bc,1 (0.38)	-0.35 (0.16)	93.8 (3.1)
	Cultivated	2.37c,1 (0.21)	89.8a,1 (0.8)	3.41c,1 (0.24)	-0.31 (0.11)	91.7 (2.9)
5–10	Forest	2.29a,2 (0.20)	91.3a,1 (1.0)	3.50a,2 (0.38)	-0.32 (0.31)	93.6 (8.0)
	Pasture	1.97a,2 (0.15)	91.9a,1 (0.5)	3.25a,2 (0.25)	-0.38 (0.21)	92.8 (5.8)
	Hayfield	1.59a,2 (0.13)	92.5a,1 (0.5)	3.43a,2 (0.29)	-0.02 (0.21)	102.3 (8.0)
	Cultivated	2.03a,12 (0.28)	90.5a,1 (0.5)	3.13a,1 (0.39)	-0.35 (0.14)	88.9 (4.7)
10–20	Forest	1.37a,3 (0.14)	91.7a,1 (1.0)	2.66a,3 (0.34)	0.13 (0.14)	102.6 (4.6)
	Pasture	1.06a,3 (0.10)	92.8a,1 (0.6)	2.50a,3 (0.15)	0.09 (0.13)	108.7 (5.7)
	Hayfield	1.14a,3 (0.10)	92.8a,1 (0.)	2.85a,2 (0.23)	0.22 (0.13)	110.8 (6.6)
	Cultivated	1.50a,2 (0.28)	90.7a,1 (0.9)	2.62a,1 (0.37)	0.11 (0.12)	104.4 (6.0)
Average of all uses	0–5 cm	3.77 (0.20)	90.9 (0.3)	5.53 (0.32)	-0.53B,S (0.11)	92.0B (1.6)
	5–10 cm	1.96 (0.10)	91.6 (0.3)	3.34 (0.16)	-0.26B,S (0.12)	94.9B (3.5)
	10–20 cm	1.24 (0.07)	92.2 (0.4)	2.66 (0.13)	0.14A,S (0.06)	107.0A (2.9)
Average of all depths	Forest	2.91 (0.30)	91.5 (0.5)	4.57 (0.48)	-0.29 (0.16)	96.2 (3.4)
	Pasture	2.29 (0.22)	92.2 (0.3)	3.83 (0.27)	-0.33 (0.12)	97.3 (3.1)
	Hayfield	2.06 (0.21)	91.7 (0.4)	3.72 (0.22)	-0.05 (0.10)	102.3 (3.7)
	Cultivated	1.97 (0.16)	90.3 (0.4)	3.05 (0.20)	-0.18 (0.08)	95.0 (3.0)

Within each depth, differences among land uses are denoted by letters. For each land use, differences among depths are denoted by numbers (Tukey-Kramer HSD;  $P < 0.05$ ). Sample numbers for forest, pasture, hayfield and cultivated sites were 11, 13, 12, and 8, respectively

<sup>a</sup> ANOVA calculated with log<sub>10</sub>-transformed data

<sup>b</sup> Significantly (S) or Non-significantly (NS) different from zero as determined by Bonferroni adjusted paired *t*-test ( $P < 0.05$ )

## Effects of different extractants on BRSP recovery

The amount of BRSP recovered by different extractants fell into two distinct classes, a high extraction group including sodium pyrophosphate, sodium oxalate and sodium citrate and a low extraction group comprised of sodium acetate, sodium formate, and sodium orthophosphate (Figs. 3, 4a). There was a significant interaction between extractant and soil depth on the % of the total BRSP extraction accounted for by the first extraction cycle ( $P < 0.0005$ ) in the high group (Fig. 5). After a single extraction cycle, the higher proportions of BRSP were extracted with sodium pyrophosphate and sodium citrate than with sodium oxalate and did not vary with depth. In contrast, extraction efficiency for sodium oxalate decreased with depth.

## Effects of tannin of extraction of BRSP

Contrary to expectations, the amount of BRSP increased after treatment with tannic acid or quebracho extracts ( $P < 0.0001$ ) (Fig. 6a). However, dark-colored compounds appeared to form in the tannin-treated samples during the extraction process that raised doubts about the accuracy of the colorimetric Bradford assay. We therefore

measured soluble-N in the soil extracts reasoning patterns of N should mirror BRSP.

Despite darker colors, less soluble-N was recovered from tannin-treated samples than untreated controls ( $P < 0.005$ ) (Fig. 6b). Average values of soluble-N recovered from 1 g. of soil treated with 10 mg tannic acid or quebracho were about 12 and 4% less than the untreated control, significant by paired-*t* test and non-parametric sign test.

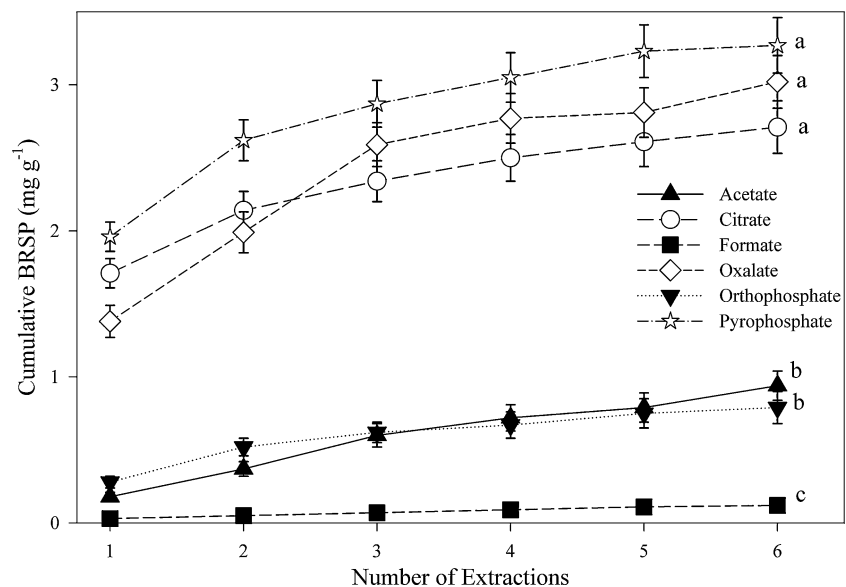
There was a significant interaction between tannin treatment and depth for E4/E6 ratios ( $P < 0.0001$ ; Fig. 7a). Treatment with tannic acid or quebracho extract decreased the ratio of absorbance at 465 and 665 nm in BRSP extracts. Lower E4/E6 ratios in BRSP extracts were found to result from either autoclaving or additions of tannin (Fig. 7b).

## Discussion

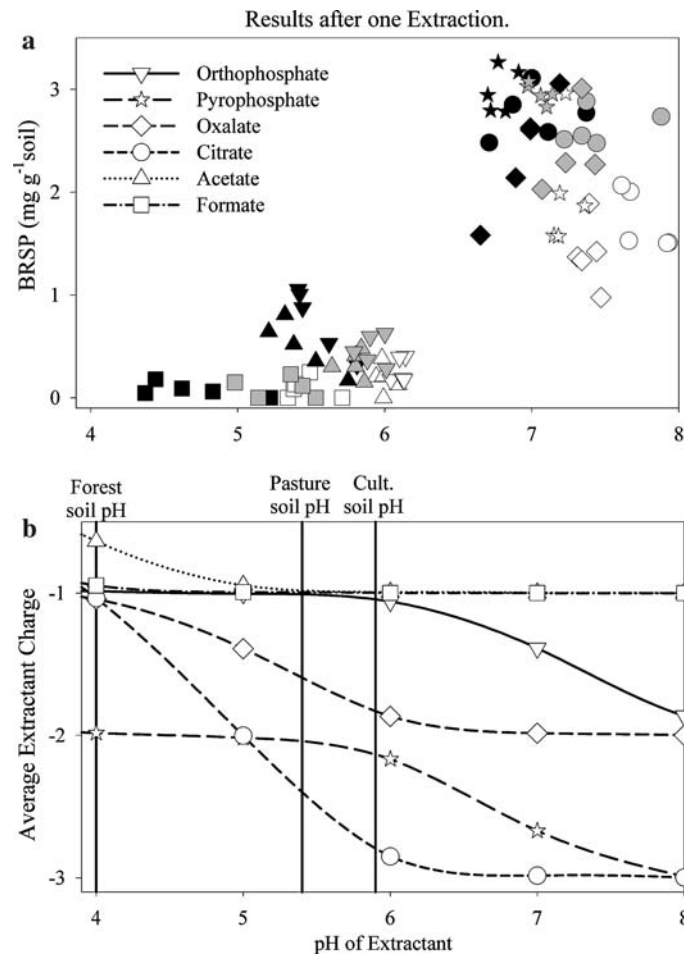
### Effects of land use and depth

Like others, we found significant quantities of BRSP present under all land uses, decreasing with depth perhaps in correlation with AMF hyphal growth (Lovelock et al. 2004b) or decreasing disturbance with depth (see below). Our overall

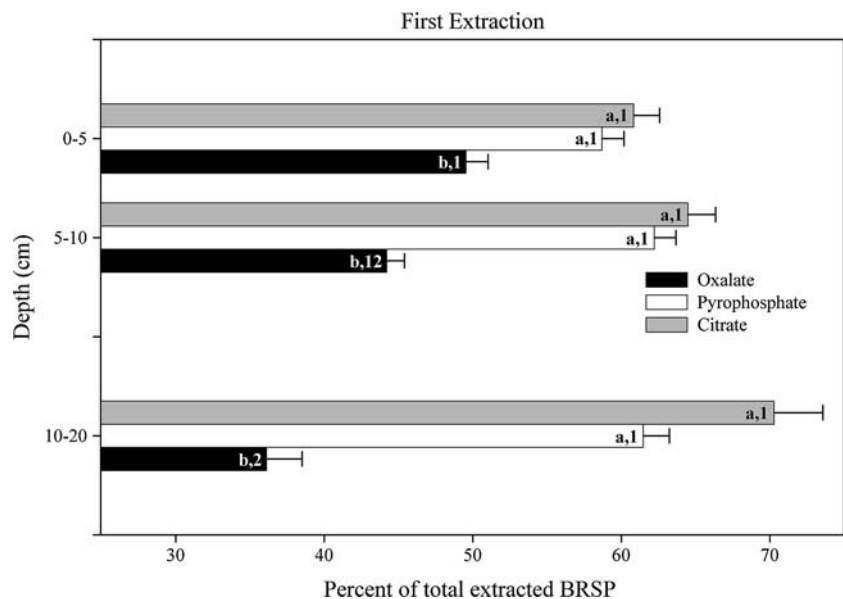
**Fig. 3** Cumulative mean (SE) extraction of Bradford reactive soil protein (BRSP) with different extractants (all depths and uses combined). Significantly different values after 6 extraction cycles are denoted by letters (Tukey's HSD  $P < 0.05$ )

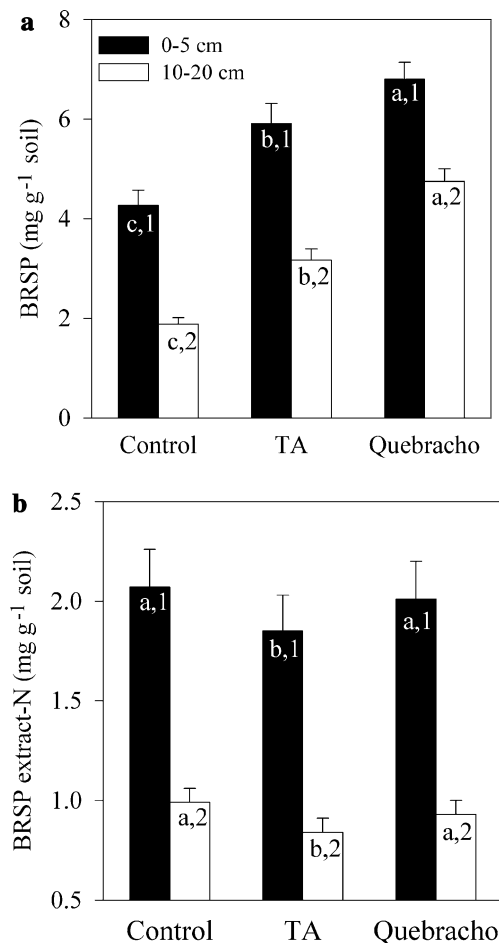


**Fig. 4** (a) The relationship between Bradford reactive soil protein (BRSP) in 0–5 cm of forest (black symbols), pasture (grey symbols), and cultivated (white symbols) samples and extractant pH after one extraction cycle, and (b) average extractant charge as a function of pH. Mean soil pH for forest, pasture and cultivated sites are shown as vertical solid lines in 5b



**Fig. 5** Mean (SE) % of cumulative Bradford reactive soil protein (BRSP) recovered after the first autoclave cycle by the high extraction group, sodium pyrophosphate, sodium oxalate and sodium citrate. Within each depth, differences among extractants are denoted by letters. For each extractant, differences among depths are denoted by numbers (Tukeys' HSD  $P < 0.05$ )





**Fig. 6** Mean (SE) cumulative (a) Bradford reactive soil protein (BRSP) and (b) soluble-N in pooled BRSP extractions. At each depth, differences among treatments are denoted by letters. For each treatment, differences between depths are denoted by numbers (Tukeys' HSD  $P < 0.05$ )

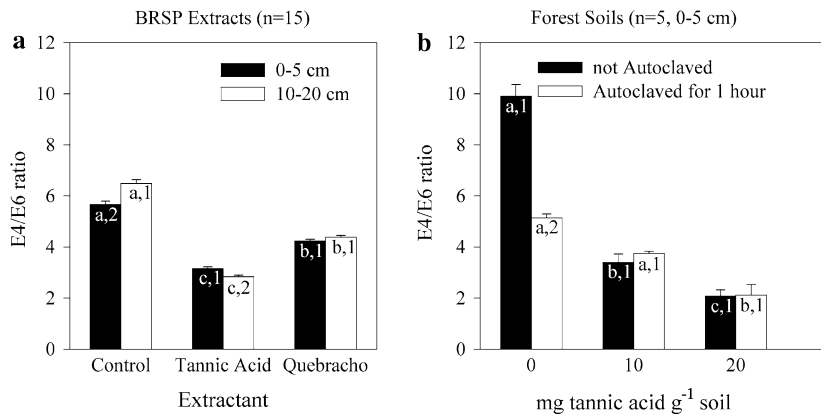
average values, 6.6 (0.3), 4.5 (0.2), and 3.4 (0.2)  $\text{mg cm}^{-3}$  soil in the 0–5, 5–10, and 10–20 cm depth increments respectively are within the range of values, 4.4–14  $\text{mg g}^{-1}$  reported for undisturbed A horizons of Mid-Atlantic forest soils (Wright and Upadhyaya 1996, 1998) and are remarkably similar to other broadly ranging data including those reported by Lovelock et al. (2004a) for the top 10 cm of tropical lowland rain forest soil (3.94  $\text{mg cm}^{-3}$ ); by Rillig et al. (2003) for the A horizons of Ohio soils under different land uses (3.79  $\text{mg cm}^{-3}$ ); by Franzluebbers et al. (2000) in 0–40 mm depth of pastures in the Southern Piedmont USA (ca. 2–4  $\text{mg cm}^{-3}$ ) and

by Harner et al. (2004) for Montana flood plains (3.38  $\text{mg g}^{-1}$ ).

Significant differences in BRSP among land uses in this study are consistent with other studies that relate increased BRSP to site or stand age (Franzluebbers et al. 2000; Harner et al. 2004; Rillig et al. 2001b), management regimes that minimize physical disruption of the soil by tillage (Borie et al. *in press*; Wright and Anderson 2000; Wright et al. 1999) or lower fertility (Lovelock et al. 2004a). Highest amounts of BRSP were observed in unfertilized forest locations with low soluble-N and pH (Table 2). However, in this study, relatively high amounts of BRSP were also found in pasture sites characterized by high amounts of soluble and total-N and intermediate pH. Grazing by livestock has been noted to have little effect on quantities of glomalin in other studies (Franzluebbers et al. 2000) and compaction by animals might even stimulate glomalin production by AMF. A reinforcing feedback between soil structure and glomalin production has been demonstrated by Rillig and Steinberg (2002) who showed glomalin production by AMF hyphae might be induced as a mechanism of habitat modification to promote aggregation. Lowest BRSP concentrations were observed for cultivated sites with relatively high soluble-N and pH.

#### Relation to soil carbon and nitrogen pools

A density dependent relationship between the C composition of lyophilized material and colorimetric estimates of BRSP concentration in the soil is suggested by Fig. 2 but alternatively may indicate C composition is a function of soil depth. This pattern, not observed for the N composition of lyophilized material, implies that glomalin or at least BRSP can exist in various degrees of C-enrichment relative to N-content (i.e., C/N ratios). Glomalin-N composition may appear more constant because the dye reagent used in the Bradford Protein Assay (Coomassie Brilliant Blue G-250) reacts primarily with N-rich amino acid residues (Bradford 1976). Glomalin is a glycoprotein, reported to be composed mostly of carbohydrate (Wright and Upadhyaya 1998), thus glomalin-C may be subject to utilization by soil microorganisms as



**Fig. 7** (a) Mean (SE) (a) E4/E6 ratios for control or tannin (10 mg of tannic acid or quebracho extract per g soil) treated samples. For each depth, differences among treatments are denoted by letters. For each tannin treatment, differences between depths are denoted by numbers (Tukeys' HSD  $P < 0.05$ ); (b) The effects of

tannic acid and autoclaving on E4/E6 ratios. For each autoclave option, differences among tannic acid treatments are denoted by letters. For each treatment, significant effects of autoclaving are by different numbers (Tukeys' HSD  $P < 0.05$ )

a function of time, depth or alternative substrate availability.

The LMS model relating the amount of BRSP in soil and carbon composition resulted in a predicted average estimate of 27.1 (0.6)% C in BRSP over all land uses and depths with a range of 17.8–57.8%. These values are comparable with those used by Rillig et al. (2003) for glomalin-C ( $27.9 \pm 3.3$ – $43.1 \pm 1.4\%$ ). However, unlike our data, Lovelock et al. (2004a) found the % C and N in lyophilized BRSP from Costa Rican soils decreased as a function of BRSP concentration. At the average BRSP concentration found in this study,  $4.06$  ( $0.19$ )  $\text{mg g}^{-1}$ , their data predict BRSP composed of at least 40% C and about 5% N. Our estimates of BRSP contributions to the total soil C and N pools, averaging  $4.1$  ( $0.1$ ) and  $6.5$  ( $0.2$ )% respectively over all land uses and depths, are comparable for C but slightly higher for N than other published data that suggest C and -N in glomalin generally represents about 3–5% of the total-C and -N pools in temperate and tropical sites (Lovelock et al. 2004a; Nichols 2003; Rillig et al. 2003, 2001b) but may be slightly higher in semiarid sites (Bird et al. 2002).

#### Changes BRSP after soil incubation

The amount and proportion of total soil C respired during a 395 day incubation in this study is comparable to the values published for a 413 day incubation of A horizon soils by Rillig et al. (2003). However, we measured a much smaller overall average change in BRSP,  $-2.1$  ( $1.7$ )%, range  $+73.5$  to  $-48.3\%$ ,  $n = 132$ , than the 48–68% reduction in BRSP they reported or the 25% decline in BRSP during a 150 day assay that Steinberg and Rillig (2003) found, equivalent to a loss rate of about 67% in a 400 day incubation. Average losses of BRSP in the 0–5 and 5–10 cm depths in this study correspond to mean residence times of 12.5–19.6 years respectively (100% divided by the rate of loss %), and fall within the 6–42 year range of minimum mean residence times for glomalin as determined by  $^{14}\text{C}$  for a Hawaiian chronosequence (Rillig et al. 2001b).

Apparent increases in BRSP in 10–20 cm samples,  $7.0$  ( $2.9$ )%, range  $+71.9$  to  $-24.7\%$ ,  $n = 44$ , were unexpected and might merely indicate small systematic differences between pre and post-incubation sample analyses or might indicate formation of some other substance, detectable by the Bradford assay, during the incubation.

Indirect evidence for another source of BRSP, besides glomalin, capable of withstanding the extraction process was reported by Lutgen et al. (2003) who found concentrations of BRSP, capable of varying seasonally by almost 25% in the top 15 cm of Montana grassland, were not correlated with AMF hyphal length or fungal activity in general. Close inspection of the data from Steinberg and Rillig (2003) indicates that BRSP losses occurred only during the first half of their 150 day incubation while constant or even slightly increasing concentrations of BRSP were evident during the second half of their incubation study.

### Effects of extractants

Use of 50 mM sodium pyrophosphate extracted about 20% more BRSP than standard 50 mM sodium citrate after 6 extraction cycles suggesting standard methods may underestimate total BRSP in soil in agreement with Wright et al. (In press). However, sodium citrate extracted a slightly higher proportion of the total after a single extraction than sodium pyrophosphate while sodium oxalate efficiency decreased with depth (Fig. 5). Changing extraction efficiencies as a function of depth, without a corresponding change in pH (Fig. 5) suggest (a) the nature of BRSP changes with depth and/or (b) the mechanism for complexing BRSP to clay minerals/humic substances changes.

Those extractants with the most negative charge extracted the most BRSP (Fig. 4a, b). As a function of pH, extractant charge can be influenced by the buffering capacity of the soil or the extractant. Extractants with small negative charge have little buffering capacity and yield little BRSP. The pH of sodium formate, acetate and orthophosphate extracts after the first extraction were most strongly influenced by soil pH (Fig. 4) whereas sodium pyrophosphate, oxalate and citrate remained closer to the original buffer pH (8.0). However, pH mediated effects on extractant charge cannot explain the decrease in extraction efficiency with depth for sodium oxalate (Fig. 5) because soil pH did not vary with depth (Table 2). Nor can they explain why similar amounts of BRSP were extracted by

sodium citrate and sodium oxalate at extractant pHs that correspond to charges of  $-3$  and  $-2$  respectively (Fig. 4b).

Glomalin is reported to be tightly bound with iron and organic matter (Nichols 2003) and charge of the extractant may determine the amount of BRSP extracted if (a) the extractants complex with the cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  that may serve as a metal bridge between the glycoproteins and the clay minerals/humic substances or (b) the extractants complex directly with iron bound to the glomalin molecule. Plants that exude weak organic acids like citrate or oxalate, a possible mechanism for increasing phosphorus availability in low pH soils may also affect the stability of SOM by affecting complexes between humic material/clays and glomalin (or other glycoproteins).

### Effects of tannin

Tannins are phenolic compounds that precipitate proteins and we hypothesized that adding tannins to soil would affect recovery of BRSP. Treatment with an aqueous tannin acid solution resulted in a darkening of BRSP extracts not seen in untreated controls. These dark colored substances may be complex, insoluble, and non-hydrolysable polymers called melanoidins. Melanoidins are N-rich, humic-like products of the Maillard reaction formed from the condensation of amino acids and reducing sugars with an affinity for clay mineral surfaces (Arfaio et al. 1999, 2003; Derenne and Largeau 2001; Hedges 1978). Presence of these dark-colored substances formed during extraction may bias the results of the colorimetric Bradford assay and result in an overestimation of soil protein when tannins are present. This may partially explain why unusually high amounts of BRSP were found in Hawaiian soils despite the absence of tannins in purified glomalin (Rillig et al. 2001b). Formation of these humic-like products might also explain recovery of less soluble-N from tannin acid treated BRSP extracts than corresponding controls. The lowering of E4/E6 ratios after the addition of either tannic acid and quebracho can be associated with the formation of larger or heavier molecules (Chen et al. 1977) and is

affected by both tannin concentration and autoclaving (Fig. 7b). Together with recovery of less soluble-N, lower E4/E6 ratios suggest tannins may form non-extractable N-containing complexes that may include glomalin.

## Conclusions

This study, together with the observations of others, suggests BRSP accounts for about 5% of total soil-C and -N and is apparently responsive to changes in land use, especially physical disturbance. This pool may indeed contain glomalin or other glycoproteins and the Bradford assay is easy to use, sensitive and has been found to produce good sample replicability in our lab. However, variations in C and N composition of recovered BRSP, different extractant efficiencies affected by soil pH and depth, variable mineralization patterns ranging, in this study, from +73.5 to –48.3% for individual samples, and possible interference from common plant secondary compounds like tannins all urge caution in accepting BRSP data as a measure of glomalin even when combined with measurements of immunoreactivity. Our data suggest BRSP is a recalcitrant pool of SOM in Appalachian soils that will be found in relative abundance at the soil surface and thus likely affected by silvopastoral management. More information is now needed to determine the interactions between polyphenolic plant secondary compounds and BRSP in soil and its role as a recalcitrant reservoir of nutrients and SOM.

## Disclaimer

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